EFFECT OF OXIDATIVE STRESS ON THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*

by

PRIYANKA SRIVASTAVA

(Under the Direction of Michael W.W. Adams)

ABSTRACT

A superoxide reduction pathway has been proposed to be involved in the oxidative stress defense in the hyperthermophilic archaeon *Pyrococcus furiosus*. This pathway includes superoxide reductase (SOR), rubredoxin (Rd), NADPH: rubredoxin oxidoreductase (NROR) and rubrerythrin (Rr). One of the goals of this study was to investigate if any of the proteins involved in SOR pathway directly interact with each other. The results indicated that this is the case in the cell free extract. The genome wide oxidative stress response of the organism to cumene hydroperoxide (CHP), an organic peroxide, was also investigated in this study. The results suggest that there are two types of responses to CHP. The constitutive response involves genes from the SOR pathway together with alkyl hydroperoxide reductase, an enzyme that utilizes CHP as one of its substrates. In addition, there is an inducible response and involves a large number of genes of unknown functions.

INDEX WORDS: Superoxide reductase, archaeon, hyperthermophile, oxidative stress, *Pyrococcus furiosus*, Rubredoxin, NADPH: Rubredoxin Oxidoreductase, Rubrerythrin, Cumene hydroperoxide

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DEDICATION

This dissertation is dedicated to my Guru:

Swami Purushottamanand ji maharaj

And

Swami Chaitanyanand ji

For

their blessings.

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CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

Archaea are the third domain of life to be recognized and includes organisms that can thrive in extreme habitats. These environments include extremes of temperature, pH and salinity and many of these organisms are also strict anaerobes. The domain archaea includes hyperthermophiles (which can grow in extremes of temperature), acidophiles (which can grow in extremes of acidity), extreme halophiles (which can grow optimally above 3.4-5.1 M NaCl), and methane-producing methanogens (1). Hyperthermophiles are usually defined as the organisms that have an optimal growth above 80 ºC and a maximum growth temperature above 90 ºC (2). They have been isolated from a wide variety of habitats, such as black and white smokers, hydrothermal vents, hot springs and solfataric fields (3). One common feature shared by all hyperthermophilic organisms is the presence of the enzyme reverse gyrase, which is responsible for creating positive supercoils in DNA (4).

The domain archaea can be divided into two major phyla, the Crenarchaeota and the Euryarchaeota (*5-7*). The Crenarchaeota contains the most abundant archaea in marine environments and comprise of thermophilic, hyperthermophilic and mesophilic species. The Euryarchaeota are phenotypically and metabolically diverse and contains halophiles, methanogens, thermoacidophiles and hyperthermophiles (5). The Nanoarchaeota and the Korarchaeota phylum are recently added to the archaea

domain. The Nanoarchaeota contains only one species *Nanoarchaeum equitans* (*6*), and Korarchaeota is based only on environmental DNA sequences with no cultured members until now (8).

The model organism of this study is *Pyrococcus furiosus*. It belongs to order Thermococcales of the phylum Euryarcheota. In the genus *Pyrococcus, P. furiosus* was the first isolated species (9). *P. furiosus* was isolated from geothermally heated marine sediments off the coast of Vulcano, Italy by Stetter and coworkers in 1986 (9). It is an obligate anaerobe with an optimal growth temperature of 100 ºC. *P. furiosus* is coccoid in shape and possesses monopolar peritrichous flagella (Fig. 1.1). It is a strict anerobic heterotroph which grows on peptides and carbohydrates (9-10). It generates hydrogen, carbon dioxide and acetate as metabolic end products. It also generates hydrogen sulfide in the presence of elemental sulfur (11). The genome has been sequenced, and 2,198 ORFs have been annotated in its genome (12-13). The organism has been well studied, with a significant amount of data available from enzymatic and metabolic studies, protein structural investigations and transcriptional analysis using DNA microarrays. (11, 14) (15-16).

The appearance of molecular oxygen on the earth started ~2.5 Gyr and it began to accumulate in the earth's atmosphere. This accumulation led to the evolution of efficient and high energy yielding processes like photosynthesis and aerobic respiration which were essential for the development of most life forms on the planet.

Most microorganisms have limited tolerance to molecular oxygen (17). There are obligate anaerobes and microaerophiles, which cannot survive in oxygenated media

and some aerobes which grow poorly when exposed to high amounts of oxygen (18). Molecular oxygen in its ground state has two unpaired electrons and is paramagnetic. Consequently, it cannot interact with other molecules unless “activated”.

The univalent reduction of molecular oxygen leads to the formation of reactive oxygen species (ROS), such as the superoxide radical ($O_2^{\cdot-}$), the hydroxyl radical ($HO^{\cdot}$), singlet oxygen ($^1O_2$) and hydrogen peroxide ($H_2O_2$) in all photosynthetic and respiratory organisms. Auto-oxidation of flavins and thiols and exposure to UV light are other ways of generating ROS (19). Gerschman and Gilbert in 1954 (20) proposed that free radicals are responsible for the toxic effects of molecular oxygen. For all biological systems, the production of ROS is directly related to the concentration of oxygen in the system (19).

These ROS have the potential to damage DNA, lipids, and proteins. When the production and accumulation of ROS exceeds the ability of the organism to remove and repair their damage, ROS imposes oxidative stress. The amount of oxidative stress imposed on an organism depends on the rate of production of hydroxyl radical and hydrogen peroxide inside the cell (17-19).

*Superoxide radical.* The donation of one electron to molecular oxygen by an electron donor leads to the formation of the superoxide radical. It can either act as an oxidant or

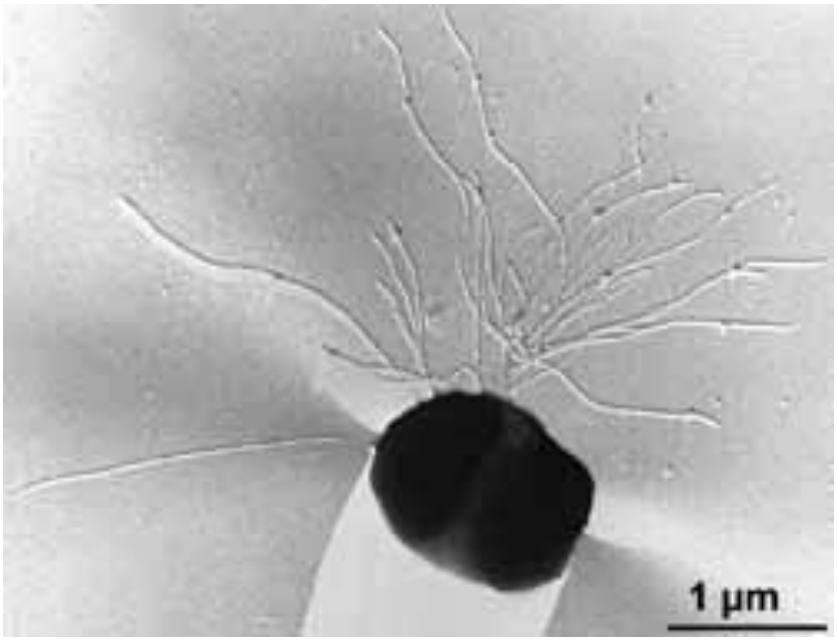


**Fig. 1.1**. An electron micrograph of *P. furiosus* showing its monopolar peritrichous flagella. Taken from (21).

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a reductant in biological systems. It has a lifetime of 50 μs and a diffusion distance of ~ 320 nm (17). The strong anionic charge of superoxide radical limits is reactivity with electron rich molecules like nucleic acids, but metal clusters within proteins are highly susceptible to oxidation by superoxide.

*Singlet oxygen:* Singlet oxygen is generated by several photochemical and chemical pathways in biological systems. When a molecule absorbs light of a specific wavelength, it moves to a higher energy state. This energy can be passed to molecular oxygen which generates singlet oxygen when the molecule returns to its ground state. It has a lifetime of 3.7 μs in aqueous media and a diffusion distance of ~82 nm (17).

*Hydrogen peroxide.* Hydrogen peroxide is produced by the dismutation of superoxide radical. It is uncharged and can easily diffuse across biological membranes. It has a half life of 5 days in oligotrophic open sea water (16). It acts as a powerful oxidant, and its potential targets of oxidation are iron –sulfur clusters, sulfur atoms of cysteine and methionine residues causing detrimental effects on the cell. In addition, the interaction of free Fe (II) with hydrogen peroxide gives rise to hydroxyl radical (19, 22) through the Fenton reaction (Eqn.1)

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^- \qquad (1)$$

*Hydoxyl radical.* It is the most reactive oxygen radical and is generated by the Fenton's reaction (Eqn. 1). It has a half life of $10^{-7}$ s and a mean diffusion distance of 4.5 nm. It has the potential to cause severe biological damage as it can initiate free radical chain reactions, damage proteins and nucleic acids, and oxidize membrane lipids (17). Of all

the ROS produced, the hydroxyl radical is the most damaging of all as it oxidizes most biological molecules at diffusion limited rates.

Oxidative stress in Archaea

The domain archaea includes organisms that can thrive in extreme habitats. Survival in these extreme environments requires them to be capable of sensing the subtle changes in their environment and to be able to either respond to or adapt to these changes (23). Biological changes that can cause change in environment include temperature and availability of oxygen.

Oxidative stress is a universal problem that influences both aerobic and anaerobic archaea. Oxidative stress studies have been reported in various methanogenic archaea like *Methanosarcina barkeri, Methanothermobacter thermoautotrohicum* (24), hyperthermophillic archaea like *Pyrococcus furiosus* (16, 25)*, P. horikoshii* (26), *Sulfolobus solfataricus* (27-28) and halophillic archaea like *Halobacterium halobium* and *H. cutirubrum*. The molecular mechanisms by which archaea respond to oxidative stress have not been studied fully and only partially understood.

Scavengers of hydrogen peroxide

Hydrogen peroxide is removed in most aerobic and anaerobic archaea by hydroperoxidases, which includes catalases (29-30) and peroxidases (23), peroxiredoxins and Dps-like proteins (DNA binding proteins first identified in nutrient starved *E. coli* cells).

*Hydroperoxidases.* This is a group of heme containing proteins which are responsible for removing hydrogen peroxide. It consists of two classes of proteins, catalases and peroxidases. Catalases convert hydrogen peroxide to oxygen and water (Eqn. 2).

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (2)$$

Peroxidases catalyse the oxidation of various organic compounds by hydrogen peroxide (Eqn. 3) using a variety of electron donors.

$$H_2O_2 + RH_2 \longrightarrow 2H_2O + R \qquad (3)$$

Catalase plays an important role in protecting cells against oxidative stress along with superoxide reductase (SOR). Catalases have been widely distributed among aerobic and facultative anaerobic organisms. It has been reported that there are two different kinds of catalases, heme catalases and non-heme catalases (31) .

*Heme catalases.* Among methanogenic archaea, catalases have been reported from *Methanosarcina barkeri* (32) and *Methanobrevibacter arboriphilus* (33). Enzyme activity and gene expression of catalase were upregulated in presence of paraquat (160 µM) and hydrogen peroxide (1.3 mM) in *M. barkeri.* Specific activity of catalase was found to be 1.5 times higher and gene expression of the *kat* gene increased five-fold when *M. barkeri* was exposed to hydrogen peroxide as compared to paraquat. This could be because hydrogen peroxide is the direct substrate for catalase while paraquat may have a very specific effect on the cells (30) .

In the halophilic archaea *H. halobium* (34-35), a catalase as well as a catalase-peroxidase has been purified. The catalase activity was found to be highest under acidic conditions, and peroxidase activity increased with an increase in pH of intracellular

environment of the cell. Among hyperthermophilic archaea, a catalase-peroxidase has been reported from *Archaeoglobus fulgidus* (36) but no catalase orthologs are found in the genome sequences of *Pyrococcus* species, including *P. abysii, P. horikoshii and P. furiosus*.

*Non-heme catalases.* Non-heme catalses utilize Mn in their active site instead of ferric iron. To date only one Mn-containing catalase has been reported from archaeon *Pyrobaculum calidifontis* (37) among archaea. *P. calidifontis* is a facultative anaerobic archaeon, and catalase activity was detected when it was grown under aerobic conditions (23) . Other aerobic hyperthermophillic archaea like *S. solfataricus* (38) and *Aeropyrum pernix* (39) do not have catalase orthologs in spite of harboring SOD, an enzyme that generates hydrogen peroxide.

*Peroxiredoxins.* Peroxiredoxins (Prx), commonly known as alkyl hydroperoxide reductases, are a group of enzymes that act as thiol peroxidases to reduce hydrogen peroxide and alkyl hydroperoxides to water and the corresponding alcohol, respectively (40). They utilize the thioredoxin (Trx) and thioredoxin reductase (Tr) system as an electron donor. Prx are divided into two groups: 1-cys Prx and 2-cys Prx on the basis of the presence of one or two conserved cysteine residues (41-43). While catalases also remove hydrogen peroxide, the difference in the working of catalase and Prx lies in their response to various concentrations of hydrogen peroxide. Catalases remove high concentrations of hydrogen peroxide, while Prx acts on low levels of hydrogen peroxide. Prx probably act as first line of defense against endogenous hydrogen peroxide as suggested by low $K_m$ values for peroxide as compared to catalase.

A novel hexadecameric Prx belonging to 2-cys Prx group has been characterized in strict aerobic hyperthermophile A*. pernix* (43)*.* It also utilizes the Tr and Trx system for hydrogen peroxide reduction. When A*. pernix* cells were exposed to hydrogen peroxide, an increase in expression of A*. pernix* Prx was detected (44).

Another archaeal Prx has been characterized from the hyperthermophile *P. horikoshii* (26) , and homologs of both 1-cys and 2-cys Prx have been reported in *P. furiosus* genome (16, 45). In a recent report published on *P. furiosus,* the gene encoding Prx was found to be upregulated within 30 min of hydrogen peroxide-induced oxidative stress (16). In *P. horikoshii,* the Prx gene showed a significant up regulation within 30 min when cells were grown under aerobic conditions and also when hydrogen peroxide (1mM) was added to the medium. This suggests that Prx in *P. horikoshii* is induced in response to aeration and peroxide. SOD and catalase are both absent in the genome of *P. horikoshii,* the presence of Prx may play a role in protecting it from oxidative stress (26).

Three homologs of Prx have been reported recently from the aerobic hypethermophilic archaeon *Sulfolobus sulfataricus* (46). They have been annotated as Bacterioferritin comigratory protein (Bcp). Bcp 2 has already been characterized, and it is known to be involved in the oxidative stress response. The transcription levels of Bcp 2 increased in the presence of various oxidative stress causing agents. Different kinetics was observed in response to different stress causing agents, suggesting that an array of regulatory mechanisms may be involved. Three additional homologs have been reported recently, Bcp1, 3 and 4 (42). All the three have the ability to scavenge both hydrogen peroxide

and tertiary-butyl hydroperoxide (t-BOOH). Bcp 1, 3 and 4 were more efficient when hydrogen peroxide was used as a substrate rather than t-BOOH as higher concentrations of BCP 1, 3 and 4 were required to remove t-BOOH. When *S. solfataricus* cells were incubated with paraquat (0.1 mM), hydrogen peroxide (0.05 mM) and t-BOOH (0.05 mM), no significant increase in the mRNA levels of Bcp1and 4 was observed. However, a five-fold increase in expression of Bcp 3 was measured 15 min after adding hydrogen peroxide. This increase in expression of Bcp 3 was comparable to that of Bcp 2, suggesting a potential role of Bcp 3 in antioxidant defense system. Prx in *S. solfataricus* utilize protein disulfide oxidoreductase (PDO)/Tr/NADPH system as an alternative electron donor system as compared to Trx/ Tr/NADPH system in a related hyperthermophilic aerobe *A. pernix.*

*Dps-like proteins.* Hydrogen peroxide produced by SOD and superoxide reductase is a powerful oxidant. It can oxidize iron–sulfur clusters and the sulfur atoms in cysteine and methionine residues. The most lethal effect of hydrogen peroxide is through the Fenton reaction, when hydrogen peroxide reacts with free Fe (II) and generates hydroxyl radical. This hydroxyl radical can attack DNA. Dps like proteins can mineralize iron within the interior of a protein cage utilizing hydrogen peroxide as an oxidant. Thus, they prevent the formation of hydroxyl ions by removing the two components of Fenton reaction. Iron mineralization in Dps is distinct from that of ferritins in that Dps utilizes hydrogen peroxide as an oxidant while ferritin uses oxygen as an oxidant.

Dps like proteins have been characterized from two hyperthermophilic archaea, the aerobe, *S. solfataricus* and the anaerobe *P. furiosus* (47-48). Dps-like proteins from

both of these organisms assembles into dodecameric cage-like structure and efficiently oxidizes Fe (II) to Fe (III) and thus prevent the formation of hydroxyl radicals. The productions of Dps-like proteins were also found to be up-regulated in response to hydrogen peroxide stress (16, 48). Dps knockout mutants have been found to be more sensitive to hydrogen peroxide-mediated oxidative damage. Dps-like proteins serve as an antioxidant defense system only in response to hydrogen peroxide and not under general stress conditions.

Scavenging superoxide radical

Superoxide radical is removed by two types of enzyme, superoxide dismutase and superoxide reductase (also known as neelaredoxin and desulfoferredoxin).

*Superoxide dismutase.* Superoxide dismutase (SOD) represents one of the most ubiquitous anti-oxidant defense systems in aerobic organisms and is also found in a limited number of anaerobes (19, 31). It catalyses the disproportionation of superoxide radical to hydrogen peroxide and molecular oxygen (Eqn. 4).

$$2O_2^- + 2H+ \rightarrow H_2O_2 + O_2 \qquad (4)$$

SOD is expected to be present in aerobic archaea and is less likely to be found in anaerobic archaea as it generates molecular oxygen during its reaction. There are four kinds of SODs which differ in the metal requirement at their active site. They are Cu/Zn SOD, Fe-SOD, Mn-SOD, and Ni-SOD. Fe-containing SODs have been characterized in many methanogenic archaea like *Methanobacterium bryantii*, *M. thermoautotrophicum*, *M. barkeri*, *Methanobrevibacter arboriphilus* (49), and in many hyperthermophilic archaea such as S*. solfataricus, S. acidocaldarius* and *Thermoplasma acidophilus.*

SOD from *S. acidocaldarius* is one of the most thermostable enzymes known so far. SOD has also been characterized from the related organism *S. solfataricus*. This SOD is inactivated by hydrogen peroxide but is insensitive to the common SOD inhibitor cyanide. Its expression is induced by oxygen in the culture fluid when *S. solfataricus* was grown in glucose–rich medium. The cell-associated SOD is also reported to maintain the integrity of outer cell-envelope components (50).

SODs from strictly anaerobic methanogenic archaea like *M. bryantii* and *M*. t*hermoautotrophicum* have been reported. Both enzymes are resistant to hydrogen peroxide and showed partial inhibition to cyanide. It has been reported that in *M. barkeri,* expression of the SOD genes were upregulated four and five-fold when oxidative stress causing agents, like hydrogen peroxide and paraquat, were used, respectively. SOD activity of M. *arboriphilus* was found to be two times higher than that of *M. barkeri. M. barkeri* is five times more sensitive to paraquat than *M*. *arboriphilus,* confirming the relationship between SOD activity and aerotolerance of species (49, 51).

Many halophilic archaea have genes encoding SOD in their genomes including *H. halobium* and *H. cutirubrum.* Their expression is up-regulated when *H. halobium* is grown in presence of paraquat and increased oxygen tension.

When strictly anaerobic organisms come in contact with oxygen, reactive oxygen species like hydrogen peroxide and superoxide radical are generated, which should be immediately removed to protect the cells from their damaging defects. SOD-catalase system acts as an antioxidant defense system in removing superoxide and hydrogen peroxide respectively.

Scavenging superoxide radical without superoxide dismutase

Superoxide reductase also includes proteins known as neelaredoxin and desulfoferredoxin.

*Superoxide reductase (SOR).* SOR are non-heme iron proteins that catalyze the reduction of the superoxide radical to hydrogen peroxide (Eqn. 5) (22, 52-55).

$$e^- + O_2^- + 2H^+ \longrightarrow H_2O_2 \qquad (5)$$

SOR was first isolated and characterized in the hyperthermophilic archaeon *P. furiosus* (*53*). SOR is one of the key components of the proposed anti-oxidant defense system in this organism. According to this pathway (Fig 1.2), SOR reduces the superoxide radical to hydrogen peroxide. The hydrogen peroxide thus generated is reduced to water by another non-heme iron containing protein termed rubrerythrin (56). This SOR reaction pathway provides an advantage to anaerobic organisms in that it does not generate the molecular oxygen.

Transcriptional analyses of *P. furiosus* have indicated that SOR and rubrerythrin are amongst the highly expressed genes when *P. furiosus is* grown under non-oxidative stress conditions, indicating that they are constitutively expressed. When hydrogen peroxide was added to *P. furiosus* culture media, there was no significant up-regulation of SOR suggesting that SOR is constitutively expressed and further up-regulation under oxidative stress conditions is highly unlikely (21, 51).

From the anaerobic hyperthermophile, *Archaeglobis fulgidus*, proteins named neelaredoxin (Nlr) and desulfoferredoxin (Dfx) have been characterized. These proteins

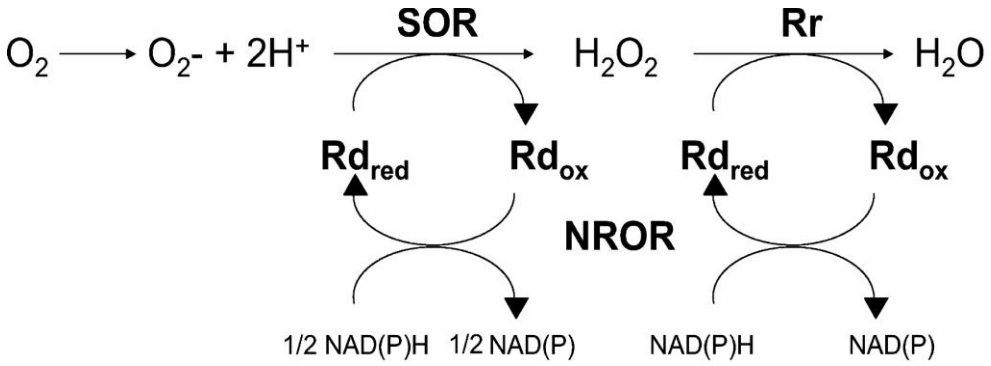



**Fig. 1.2.** Proposed oxidative stress defense pathway in *P. furiosus.* Adapted from (56).

have the same non-heme iron center as SOR and are also involved in scavenging superoxide radicals. *A. fulgidus* is the only known anaerobe so far whose genome encodes genes for both Nlr and Dfx. Other anaerobes have only one of these superoxide-scavenging proteins. Dfx acts as an SOR while Nlr acts as a bifunctional enzyme and has both dismutase and reductase activity. Recently, a mechanism by which these proteins detoxify superoxide radical was proposed (57-58). This SOD activity of Nlr is relatively low compared to that of canonical SODs, but the higher concentration of Nlr compensates for its lower activity. The bifunctional behavior of Nlr provides a protection mechanism to cells that is independent of redox status of the cell.

Goals of study

A SOR pathway involved in eliminating ROS has been proposed in *P. furiosus.* The proteins involved in this pathway are SOR, rubrerythrin, NADPH: rubredoxin oxidoreductase (NROR) and rubredoxin. All of these proteins have been isolated and purified individually through multistep chromatography of extracts of *P. furiosus* cells. The first goal of this study was to investigate if any of these proteins directly interact with each other within the cell.

In addition, to date, there have been no published reports of the oxidative stress response to an organic peroxide on a genome-wide basis in any archaeal species. The second goal of this study was therefore, to determine the transcriptional response to oxidative stress using cumene hydroperoxide in the hyperthermophilic archaeon *P. furiosus.*

CHAPTER 2

# A MULTIPROTEIN OXIDATIVE STRESS COMPLEX COMPRISING SUPEROXIDE REDUCTASE AND RUBRERYTHRIN FROM THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*

*Pyrococcus furiosus* is an obligate anaerobe which inhabits shallow marine volcanic vents and has an optimal growth temperature of $100^0$C. These vent fluids contains high amounts of sulfide and are hot and anaerobic. Mixing of these vent fluids with cold and oxygenated sea water causes a decrease in temperature and expose *P. furiosus* to oxygen (19). Reactive oxygen species (ROS) like the superoxide radical, hydrogen peroxide and the hydroxyl radical are formed as a result of incomplete reduction of molecular oxygen and impose oxidative stress on *P. furiosus*. Oxygen and sulfur centered radicals are also formed as a result of spontaneous oxidation of hydrogen sulfide near the vents that ultimately forms hydrogen peroxide and imposes oxidative stress on *P. furiosus*. Consequently, it is very likely that *P. furiosus* has a defense system that can protect itself from temporary exposures to oxidative stress and lower temperatures.

*P. furiosus* contains SOR which reduces superoxide to hydrogen peroxide (53). It has been proposed that the monomeric Fe- containing redox protein, rubredoxin (Rd) is reduced by the enzyme NADPH: rubredoxin oxidoreductase (NROR) using NADPH as

an electron donor, (51, 53, 56). In this pathway (shown in Fig 2.1), it is proposed that at least some of the hydrogen peroxide generated by SOR is reduced to water by a non-heme iron containing protein, rubrerythrin (Rr). In the process of reducing hydrogen peroxide to water, rubredoxin is reduced by the enzyme NROR using NADPH as an electron donor. This suggests that the reducing power for reduction of superoxide and hydrogen peroxide is provided by NROR via NADPH.

When *P. furiosus* was grown under non-oxidative stress conditions, transcriptional analysis using DNA-microarrays indicated that genes encoding SOR and Rr were amongst the most highly expressed genes (14). Also, the genes encoding rubredoxin and rubrerythrin are adjacent to gene encoding SOR suggesting that they may be co-transcribed (53). All of the above mentioned proteins have also been purified individually using multistep chromatographic analysis (51, 53, 56, 59). The purpose of this study was to investigate if any of the proteins involved in proposed oxidative stress defense pathway in *P. furiosus* including SOR, Rd, Rr and NROR interact with each other.

Materials and Methods:

*Polyclonal antibodies against SOR, Rd, Rr and NROR*:Polyclonal antibodies against recombinant SOR, rubredoxin, rubrerythrin and NROR were generated from the Polyclonal Antibody Services, University of Georgia. The recombinant forms of the proteins were kindly provided by Dr. Frank Jenney and Dr. Angeli Menon. Recombinant SOR and recombinant ruberythrin were used for antibodies production using concentration of 500 µg/ml and 200 µg/ml in 50mM Tris, pH 8 respectively.

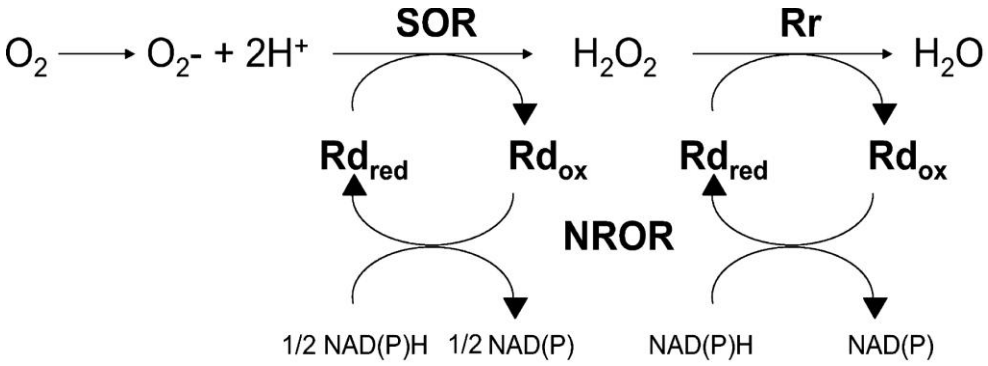



**Fig. 2.1.** Proposed oxidative stress defense pathway in *P. furiosus.* Adapted from (56).

Rabbits were given a primary injection of the antigen and a booster dose was given two weeks later. Three booster doses were given in total for each antigen and after that bleeds were collected which contained the antibodies.

IgG from SOR-antiserum was purified using Protein A resin. The column (1 ml) was equilibrated with 5 ml of Binding buffer A containing 20 mM $Na_2HPO_4$ and 0.15 mM NaCl, pH 7.0. The SOR-antiserum (0.5 ml) was diluted 1:1 with binding buffer A and loaded onto the column which was washed with 30 ml of binding buffer A. IgG was eluted using Elution buffer B (15 ml) containing 0.1 M citric acid, pH 3.0. IgG was collected in 500 µl fractions and immediately neutralized with 100 µl 1M Tris-HCl, pH 8.5. Protein estimation of the eluted IgG fractions was carried out using the Bradford's assay with IgG as standard. The same procedure was repeated to purify IgG from Rr antiserum.

*Preparation of P. furiosus cell extract.* Frozen cells (5 g) of *P. furiosus* cells grown with maltose as the carbon source were thawed anaerobically in 500 mM anaerobic Tris-HCl, pH 8.0 (15 ml). DNase was added and cells were incubated at 37ºC for 30 min. Cells were lysed by sonication for 10 min and whole cells were removed by centrifugation at 10,000xg for 15 min at 4ºC. The cytoplasmic fraction was obtained by centrifugation at 100,000xg for 1 hr. The supernatant (S100) was removed and stored at -20 ºC for future use. Protein estimation in the S100 fractions was performed using the Bradford assay with bovine serum albumin as the standard.

*Immunoprecipitation analysis.* This was performed using the Seize X Protein A Immunoprecipitation kit from Pierce chemicals (Catalogue # 45215). The S100 (2 mg)

was incubated at 4ºC overnight with 50 µl of protein A beads and 10 µl rabbit preimmune IgG. The supernatant was collected and used for the Immunoprecipitation reaction. Protein A slurry (0.4 ml) was added to a Handee Spin column placed inside a Handee microcentrifuge tube and centrifuged at 3000xg for 1 min. The gel was washed twice with 0.4 ml Binding/Washing buffer containing 0.14 M NaCl, 0.008 M sodium phosphate, 0.002 M Potassium phosphate and 0.01 M KCl , pH 7.4. rSOR IgG ( 25 µg) diluted 1:1 with binding/washing buffer was added to the spin cup and incubated at 4ºC for 30 min in a shaker. After washing three times with 0.4 ml binding/washing buffer the antibody bound to protein A resin was crosslinked using DSS (Disuccinimidyl suberate) and incubated at 4ºC for 60 min in a shaker. After centrifugation, the gel was washed four times with 500 µl Immunopure Elution buffer, pH 2.8 (supplied with the kit) to stop the reaction and remove any non-covalently bound IgG. Precleared S100 (2 mg) diluted 1:1 with binding/washing buffer was added to the spin column containing protein A bound IgG and incubated at 4ºC overnight. The immunoprecipitation (Ip) complex was eluted with 190 µl of ImmunoPure Elution Buffer. The Ip complex was eluted in 3 fractions and immediately neutralized with 10 µl of 1M Tris, pH 9.5. The fractions containing the Ip complex were concentrated using a vaccum centrifuge and then loaded onto two SDS-PAGE gels. After separation, one of the gels was silver stained and the other was transferred to a nitrocellulose membrane and probed with anti-SOR IgG. The same experimental protocol was used with anti-Rr antibodies and the Ip complex was collected and analysed. In both cases, the control experiment S100 incubated with rabbit preimmune IgG was utilized.

*Size exclusion chromatography*. Superdex 200 HR 10/30, 24 ml (Pharmacia Lkb Biotechnology) with a separation range from 10,000-600,000 Da was used. The column (24 ml) was equilibrated anaerobically with buffer A containing 50mM Tris HCl, pH 8.5 and 300 mM KCl. 300µg of recombinant SOR(56 kDa) and recombinant Rr (40 kDa ) were loaded onto the column individually or as a mixture (the SOR to Rr ratio was 1:2). Proteins were eluted in 400 µl fractions at a flow rate of 0.4 ml/min. The fractions were analysed by SDS-PAGE and western blotting using SOR and Rr antiserum.

Results

To study the protein–protein interactions involved in the oxidative stress defense in *P. furiosus*, polyclonal antibodies were raised against recombinant form of SOR, Rd, Rr and NROR. The antibodies raised against rSOR and rRr were found to be specific as indicated by a single band at the expected molecular weight for each protein after probing the SDS-gel of the S100 fraction with SOR and Rr-antiserum. In contrast, antibodies to rRd and rNROR gave multiple intense bands. Therefore, only SOR and Rr antibodies were used for the immunoprecipitation experiments.

SOR and Rr immunoprecipitation experiments were performed using the Seize X Protein A Immunoprecipitation kit. Each immunoprecipitation complex (Ip) was loaded onto a SDS-PAGE gel and analyzed by silver staining (Fig. 2.2) and immuno blotting (Figs. 2.3 and 2.4). Because of the low concentration of the Ip complexes, the proteins interacting with SOR and Rr could not be identified by silver staining and by MALDI-TOF analysis. The red arrows (Fig. 2.2) indicate the presence of faint bands at 15 and 20 kDa suggesting the presence of SOR and Rr in their respective Ip complexes.

Immunoblot analysis using SOR antisera and Rr antisera were performed to determine the presence of SOR and Rr in their respective Ip complexes. Immunoblot analysis using SOR-antisera (Fig. 2.3) revealed the presence of a 14 kDa band (lanes 3 and 4). This shows the presence of SOR in SOR Ip as well as in Rr Ip. When the same SOR and Rr Ip complexes were analyzed by immunoblotting using Rr antiserum (Fig. 2.4), a 20 kDa band was identified (lanes 1 and 2). This result suggests that Rr is also present in SOR Ip as well as in Rr Ip complexes. When similar experiment was performed with S100 incubated with rabbit preimmune antiserum, no bands were detected at 14 kDa and 20 kDa. The immunoblot analysis suggests that SOR and Rr interact with each other.

*Size Exclusion Chromatography*. The S100 fraction was analysed under non-denaturing conditions by size exclusion chromatography using a Superdex 200 HR 10/30 column. The column was equilibrated anaerobically with buffer A containing 50 mM Tris pH 8.5 and 300 mM KCl and the fractions were analysed by SDS-PAGE and immunoblot analysis using SOR antiserum and Rr antiserum. The immunoblot analysis showed that both SOR and Rr started to elute from the column with an apparent molecular weight of 200 kDa and continued to be eluted until an apparent molecular weight of 12 kDa. The 200kDa value suggests that SOR and Rr form a 2:1 complex (SOR is a homo-tetramer of 56 kDa and Rr is a homo-dimer of 40 kDa). In addition, SOR and Rr co-elution corresponding to 96 kDa suggests that they also form a 1:1 complex. However, a major

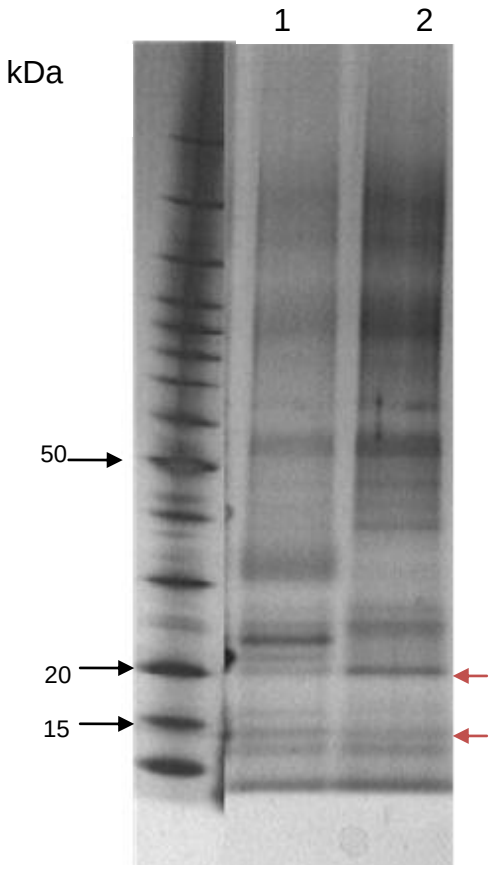



**Fig. 2.2**. Silver stained SDS-PAGE gel. The Immunoprecipitation complexes obtained with SOR and Rr antibodies were loaded onto a SDS-PAGE and analysed by silver staining. The positions of the molecular weight standards are indicated. Lane 1: Immunoprecipitation complex with SOR antisera (SOR Ip) and Lane 2: Immunoprecipitation complex with Rr antisera (Rr Ip).

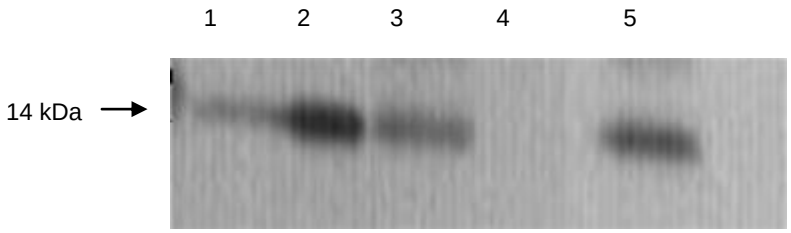



**Fig. 2.3**. Immunoblot analysis of SOR Ip. Proteins were transferred onto a nitrocellulose membrane and probed with rabbit anti-SOR antisera. The position of molecular weight standard is indicated. Lane1: S100; Lane 2: recombinant SOR (14kDa); Lane 3: SOR Ip; Lane 4: recombinant Rr (20kDa) and Lane 5: Rr Ip.

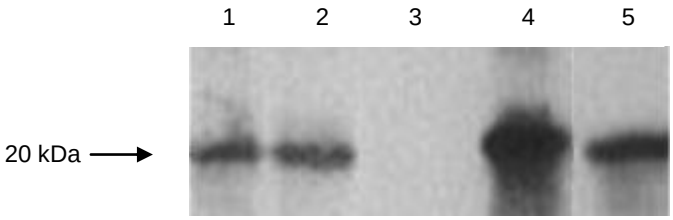



**Fig. 2.4.** Immunoblot analysis of Rr Ip. Proteins were transferred onto a nitrocellulose membrane and probed with rabbit anti-Rr antisera. The position of molecular weight standard is indicated. Lane1: Rr Ip; Lane 2: SOR Ip; Lane 3: recombinant SOR (14kDa); Lane 4: recombinant Rr (20kDa) and Lane 5: S100.

part of SOR and Rr also co-elute at lower molecular weight fractions in the range 20-12 kDa. This suggests that, most of SOR and Rr are in their holoenzyme forms and do not participate in complex formation.

Similiar size exclusion experiments were performed with recombinant SOR and recombinant Rr individually and mixed in a 2:1 ratio and incubated for one hour at room temperature. The elution profile of the rSOR and rRr mixture suggested that the pure forms of proteins do not interact. Immunoblot analysis on the eluted fractions from the rSOR and rRr mixture showed that SOR and Rr co-elute at 200kDa and 96 kDa and continued to co-elute in the lower molecular weight range. In addition, when pure SOR and Rr were mixed together and heat treated at 80 °C for one hour, there was no change in the elution profile (as compared to non-heat treatment). One of the possible reasons for elution of SOR at later than expected position (indicating a lower mol. weight than the holoenzyme) is that it may be sticking to the column.

Discussion

Rr from *P. furiosus* precipitates when oxidized by air, ferricyanide or peroxide in its purified reduced state. Since precipitation is not expected to occur *in vivo,* one of the possibilities raised were that Rr may exist *in vivo* as a multiprotein complex (56).

The results from immunoprecipition experiments using SOR and Rr antisera reported here suggest that Rr interact with SOR and form a complex. Gel filtration chromatography was used to determine the size of the complex using Superdex 200

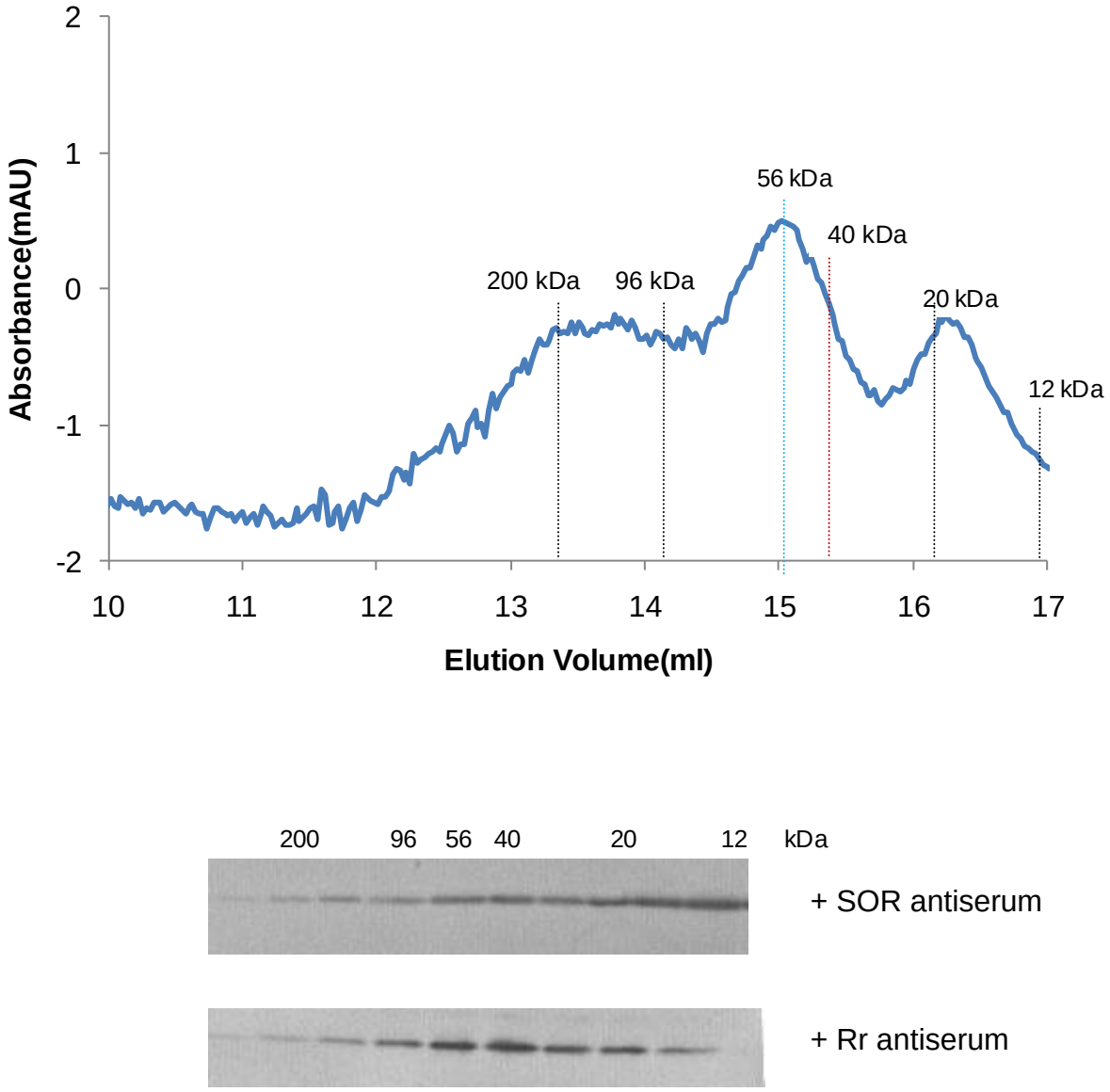



**Fig. 2.5** Elution profile of S100 (Upper). Immunoblot analysis of eluted fractions with SOR and Rr antisera (Lower). Molecular weights of the eluted fractions are indicated above the immunoblot and the elution profile.

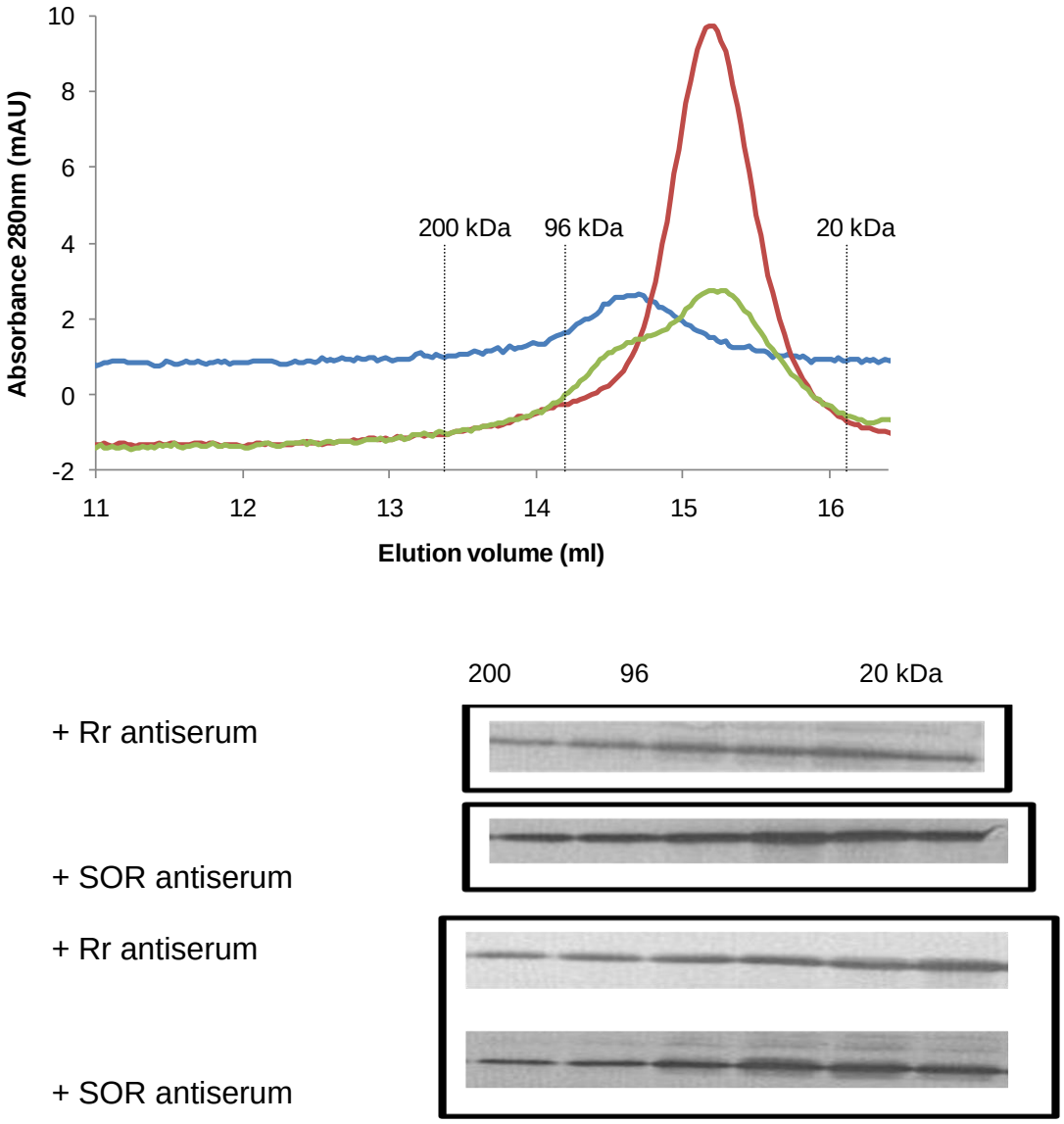



**Fig. 2.6**. Elution profile of rSOR (→), rRr (→) and SOR/Rr mixed (→)(Upper). Immunoblot analysis of eluted fractions with SOR and Rr antisera (Lower). Molecular weights of the eluted fractions are indicated above the immunoblot and the elution profile.

suggested that in the cytoplasmic extract of *P. furiosus,* SOR and Rr form a 1:1 complex of 96 kDa and may also form a 2:1complex. The results also indicate that a very small part of total SOR and Rr participates in forming the complex. We have been able to capture the SOR interacting with Rr using the immunoprecipitation experiment.

However, the SOR and Rr complex is a transient complex and not stable. The complex formation cannot be achieved reversibly when pure forms of SOR and Rr were mixed together (Fig 2.6).

Homologs of SOR and Rr have been found in many anaerobic bacteria such as *Thermotoga maritima* (60)*, Desulfovibrio vulgaris* (61) and *Clostridium acetobutylicum* (62) and archaea such as *Archaeoglobus fulgidus* (56). This is the first report of an interaction between SOR and Rr.

CHAPTER 3

# TRANSCRIPTIONAL RESPONSE TO CUMENE HYDROPEROXIDE IN THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*

Oxidative stress is a universal phenomenon that affects aerobic and anaerobic organisms alike. Production of reactive oxygen species (ROS) is the leading cause of oxidative stress in an organism. ROS are formed as a result of incomplete one electron reduction of molecular oxygen. Some of the main ROS involved in causing oxidative stress are superoxide radical ($O_2$-), hydroxyl radical (OH-) and hydrogen peroxide etc. These ROS have the potential to damage membranes, lipids, proteins, DNA and various other cellular components. Most aerobes and anaerobic organisms have therefore, evolved pathways to protect themselves from these harmful ROS. Oxidative stress has been widely studied in aerobes, and a large amount of data is available for oxidative stress defense in aerobes. In aerobes, this protection mechanism includes enzymes like superoxide dismutase (SOD, Eqn. 1) and catalases (Eqn. 2).

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (2)$$

Homologs of SOD and catalase are found in only a few anaerobes. The genomes of most anaerobes completely lack SOD and catalase, but contain superoxide reductase (SOR, Eqn. 3) and so-called Dps-like proteins (Eqn. 4).

$$O_2^- + 2H^+ + e^- \longrightarrow H_2O_2 \qquad (3)$$

$2Fe^{+2} + H_2O_2 + 2H_2O \longrightarrow 2Fe(O)OH + 4H^+$ (4)

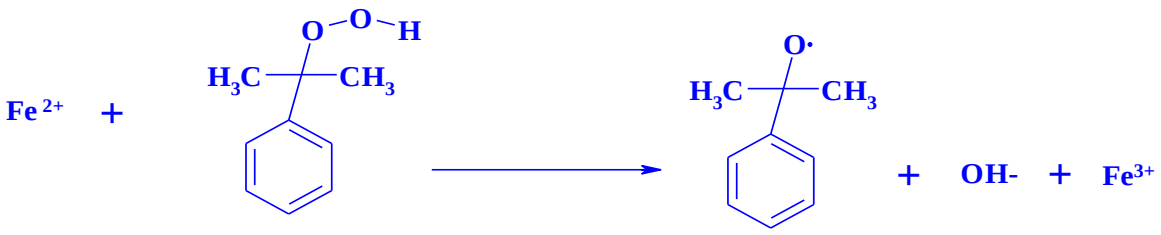
SOR not only provides protection against superoxide radical, but also provides another advantage to anaerobes as it does not produce molecular oxygen (Eqn. 3). SOR was first characterized in a hyperthermophilic archaeon *Pyrococcus furiosus* (53), which is also the model organism of this study. In *P. furiosus*, it has been proposed that the source of electrons for SOR is a monomeric Fe- containing redox protein, rubredoxin (Rd), which in turn is reduced by the enzyme NADPH: rubredoxin oxidoreductase (NROR) using NADPH as an electron donor (21). Hydrogen peroxide generated by SOR is reduced to water by a non-heme iron containing protein, rubrerythrin (Rr).

In addition, most anaerobes have alkyl hydroperoxide reductases which are involved in reducing hydroperoxides to water and alkyl hydroperoxides to corresponding alcohols. Alkyl hydroperoxide reductase has been characterized in *Salmonella typhimurium*, *Clostridium pasteurianum, Mycobacterium tuberculosis, Helicobacter pylori.* Expression of Alkyl hydroperoxide reductase from *Pyrococcus horikoshii* has been reported to be induced by oxygen and involved in peroxide detoxification (26). The expression of the gene encoding alkyl hydroperoxide reductase is upregulated under oxidative stress conditions in some anaerobes (63-64). Another protein involved in peroxide detoxification in many aerobes and anaerobes is the so-called Dps protein. Dps proteins are reported to be involved in iron sequestration and DNA–binding under oxidative stress conditions (47-48)

Transcriptional response to oxidative stress using an inorganic peroxide like hydrogen peroxide has been carried out in various anaerobes including *Porphyromonas gingivalis*

(65), *Bacteroides fragilis* (27, 66) and *Thermotoga maritima* (67) as well as in *P. furiosus* (*16*). To date, there are no available data on the transcriptional response of any anaerobe to oxidative stress using an organic hydroperoxide. The purpose of this study was to determine the effect of oxidative stress caused by the organic hydroperoxide, cumene hydroperoxide on the transcription of hyperthermophilic archaeon *P. furiosus.*

*P. furiosus* is an obligate anaerobe that inhabits shallow marine volcanic vents and has an optimal growth at 100 $^{0}$C. These vent fluids are sulfide-rich, hot and anaerobic. When these vent fluids mix with cold and oxygen-saturated sea water, *P. furiosus* encounters oxygen and a decrease in temperature. Reactive oxygen species (ROS) like superoxide radical, hydrogen peroxide and the hydroxyl radical are formed as a result of incomplete reduction of molecular oxygen and cause oxidative stress on *P. furiosus*. These hydrothermal vents can also generate other ROS (68). For example oxygen and sulfur centered radicals are formed as a result of the spontaneous oxidation of hydrogen sulfide near the vents that ultimately leads to formation of hydrogen peroxide. This study will focus on the transcriptional response of *P. furiosus* to cumene hydroperoxide (CHP). CHP is lipid soluble organic peroxide that has been used for years to study oxidative stress in various organisms.




Cumene hydroperoxide Cumyloxyl radical

**Fig. 3.1.** Structure of Cumene hydroperoxide and its reduction product after reaction with ferrous iron.

CHP is genotoxic and has the potential to induce DNA damage and mutations. Exposure of man-made fuel oil slicks to UV radiations forms CHP as a byproduct. CHP can also enter into aquatic systems from industrial discharges and spills. In the recent oil spill in Gulf of Mexico, tens of thousands of barrels of oil are leaking everyday (at the time of writing) and being exposed to UV rays. There will therefore be a tremendous increase in CHP concentration in these marine waters. In this study we will determine how a hyperthermophilic marine archaeon like *P. furiosus* responds to CHP.

Materials and Methods

*Determining the optimal concentration of CHP. P. furiosus* (DSM 3638) cell cultures were grown in 50 ml of standard anaerobic media containing 0.5% (w/v) maltose and 0.05% (w/v) yeast extract as described (69). Different concentrations of CHP were added when cultures were in mid log phase (approx. cell density of ~$5x10^7$ cells/ml). Cell growth was monitored by cell counts and protein determination using the standard Bradford's assay with bovine serum albumin as control. Cell counts were done using a Petroff-Hausser counting chamber. For protein estimations, 1 ml of cell culture was taken out and centrifuged at 14,000 rpm for 30 min and resuspended in 10 µl of deionised water and stored at -20ºC.

*Transcriptional Analysis.* 20 liters culture of *P. furiosus* was grown at 95°C using maltose as the carbon and energy source (69). 20 µM CHP was added when culture reached a cell density of ~$5x10^7$ cells/ml. To prepare RNA for microarray and quantitative PCR (Q-PCR) analysis three liters of cell culture was removed and cooled to 4 °C immediately before adding CHP. After adding CHP two liters of cell culture was

removed at 10, 15, 30, 60 and 120 min. Each culture was centrifuged at 6000 rpm for 15 min to obtain the cell pellets. RNA was extracted using acid–phenol (70).

To study the transcriptional response, PCR based microarray analysis were performed. Double-stranded PCR products containing all 2198 open reading frames as annotated in *P. furiosus* genome were printed on glass slide as described (12, 14) with the exception that there were 4 copies of each gene on one slide and purified RNA was used to hybridize with PCR–products spotted on the slide.

Two times acid–phenol purified RNA was DNAse-treated at 37°C for 20 min and then purified. The resulting RNA was fluorescently labeled with Alexa flor dyes 647, 594 and 532 as per manufacturer's instructions using spin columns. The labeled RNA was dried under vacuum and the quality of fluorescently labeled RNA was determined using Nano drop spectrophotometer. The number of dye molecules per hundred bases was determined from the Invitrogen website http://probes.invitrogen.com/resources/calc/basedyeratio.html

RNA was labeled from the CHP-shocked cells harvested at time zero (immediately before adding CHP) and at times 10, 15, 30, 60 and 120 min after adding CHP.

*Quantitative PCR*. For q-PCR, RNA purified as above was used for cDNA synthesis. cDNA was synthesized using the kit from Agilent as per manufacturer's instructions. The regulated genes as shown by microarray analysis, PF0101, PF1033, PF1072, PF1080, PF1193, PF1314, and PF2025 were investigated. The non regulated genes PF0965 and PF0971 were used as controls. The q-PCR reaction was performed as

described in (16). Stratagene's Mx3000P instrument was used for all the q-pCR analysis using Brilliant SYBR Green QPCR master mix.

Results

*The response of P. furiosus to CHP. P. furiosus* cultures grown on a small scale (40 ml) were used to determine the concentration of CHP that can cause a lag phase in growth followed by a period of recovery. Addition of 20 µM CHP resulted in some cell death followed by a recovery phase that lasted 30 min before the cells began to grow. For the transcriptional analysis, the experiment was scaled up to 20 liter fermenter and 20 µM CHP was added at mid-log phase. This resulted in some cell death followed by recovery phase (Fig. 3.1).

*Transcriptional response of P. furiosus to 20 µM CHP*. To study the transcriptional response to CHP, cells were removed at different times (10 and 30 min) from the 20 liter fermenter and RNA samples were prepared. These RNA samples were compared to RNA samples obtained before adding CHP (time zero). These three RNA samples were fluorescently labeled and hybridized to the DNA microarray. Microarray analysis revealed that out of 2,198 ORFs analyzed, 50 ORFs were regulated more than 3-fold and all were up-regulated.

*Trancriptional response within 10 min of adding CHP .*

The 25 ORFs that were up-regulated more than 3 fold are listed in Table 3.1.

*CHP related proteins. P. furiosus* genome encodes two alkyl hydroperoxide reductases (PF0722 and PF1033). Alkyl hydroperoxide reductase utilizes CHP as one of its substrate (71) and its induction is reported to be dose-dependent. Based on available

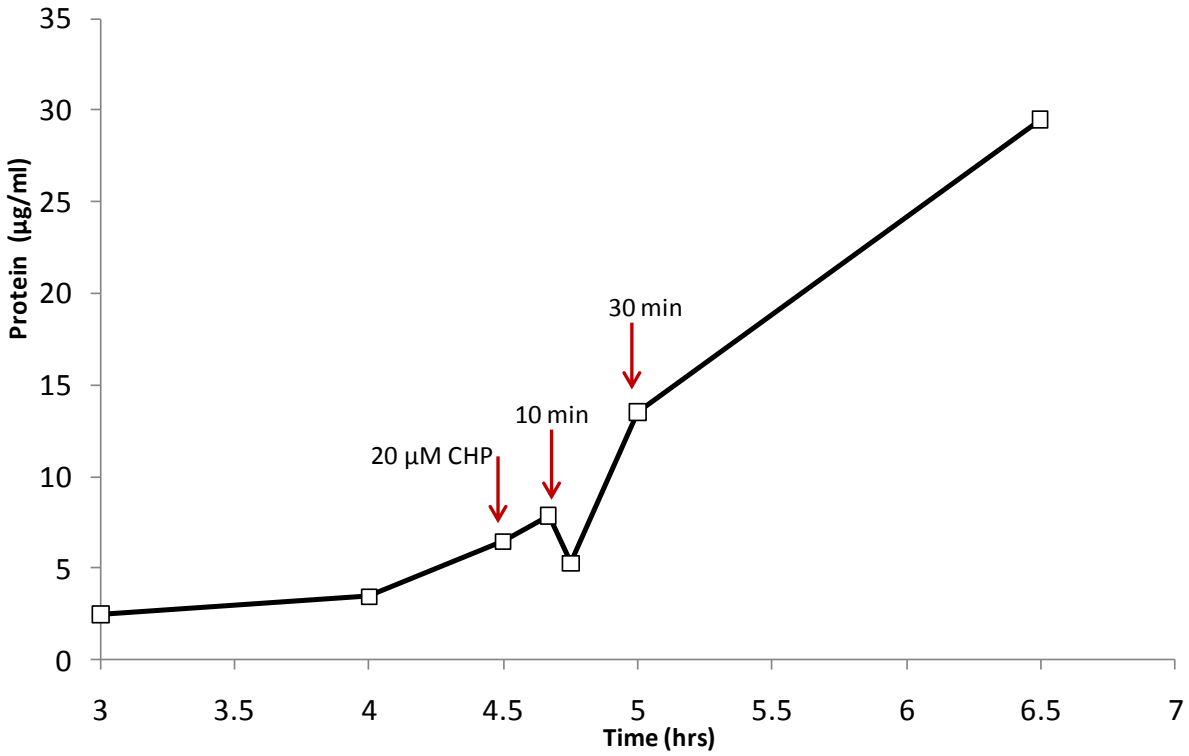



**Fig. 3.2.** Effect of CHP on the growth of *P. furiosus*. CHP (20 µM) was added when cells were in mid log phase (~5x10$^7$ cells/ml) at 4.5 hrs as indicated by the arrows. Cell growth was measured by protein concentration.

data, it was expected that the gene encoding alkyl hydroperoxide reductase (PF1033) should be regulated in response to CHP. As expected, PF1033 was up-regulated 7.5 fold according to the microarray. However, the same gene was up-regulated 107-fold by Q-PCR (Table 3.3). This suggests that in CHP shock, PF1033 is immediately turned on at high levels in an attempt to remove CHP from the system. On the other hand PF0722, was not significantly regulated in CHP shock, which can be explained by the fact that this gene is highly expressed when *P. furiosus* is grown in non-stressed conditions (70). Presumably, it cannot be further regulated in response to oxidative stress. These data are consistent with a recent report on hydrogen peroxide-induced oxidative stress in *P. furiosus* showing that PF0722 was also not regulated by hydrogen peroxide shock (16).

*Oxidative stress related genes*. Another gene that has been shown to be involved in oxidative stress in *P. furiosus* encodes the Dps protein (PF1193) (16). Dps proteins are involved in sequestering iron and prevent hydroxyl radical generation by utilizing hydrogen peroxide to oxidize Fe (II). Microarray analysis showed that PF1193 is 4.0 fold up-regulated and it is 17-fold up-regulated by Q-PCR in response to CHP shock. This gene is also up-regulated in response to iron limitation and oxidative stress in the aerobic hyperthermophilic archaeon *Sulfolobus solfataricus* (48) and also after exposing *P. furiosus* to gamma irradiation (25).

*DNA-related proteins*. CHP has been reported to cause damage to DNA by causing breaks in DNA strands. DiRuggiero et al (72) has shown that *P. furiosus* is highly resistant to gamma radiation and thus may have an efficient DNA repair system.

Microarray analysis showed that PF1926 which encodes rad A, is up-regulated 3.8-fold in response to CHP induced oxidative damage.

*Sulfur-related protein.* Only one of the many sulfur-related ORFs was regulated in response to CHP shock and this is PF2025. It is strongly up-regulated as judged by microarray analysis (7.1-fold) and by Q-PCR (29-fold). This gene encodes SipA (for Sulfur Induced Protein) and is known to be up-regulated in sulfur-grown *P. furiosus* cells. This gene is also significantly up-regulated in response to hydrogen peroxide-induced oxidative stress in *P. furiosus* (16). It may be involved in iron homeostasis based on its role in peroxide and elemental sulfur response.

*Genes of unknown function*. Of the 25 ORFs up-regulated within 10 min of adding CHP, 11 ORFs are of unknown function. They include PF0101, PF0693, PF1072-PF1074, PF1080 and PF1314-PF1315. Except PF0693, all of these ORFs are also regulated by hydrogen peroxide induced shock in *P. furiosus* (16). This suggests that these ORFs may have a role in protecting the organism against oxidative damage, although their role in oxidative stress protection is not known. To gain knowledge about their protection mechanisms, their gene products will need to be characterized, but that is beyond the scope of this study.

*Trancriptional response within 30 min of adding 20 μM CHP.*

The transcriptional response to CHP at 30 min includes 25 ORFs up-regulated more than 3-fold and they are listed in Table 3.2. They are dominated by genes of unknown function.

**Table 3.1.** ORFs whose expression is up-regulated within 10 minutes of adding CHP. Those in bold are potentially part of operons.

| PF# | Gene name | Fold |
|---|---|---|
| | Oxidative stress | |
| PF1033 | Alkyl hydroperoxide reductase | 7.5 |
| **PF1072** | Winged helix repressor DNA binding | 7.2 |
| PF1193 | Dps-like protein | 4.0 |
| | CRISPR related | |
| **PF0639** | CRISPR associated protein | 3.0 |
| **PF0640** | CRISPR associated protein | 4.8 |
| **PF0641** | CRISPR associated protein | 5.4 |
| **PF0642** | CRISPR associated protein | 9.1 |
| PF0643 | CRISPR associated protein | 7.9 |
| | DNA related | |
| PF1926 | Rad A | 3.8 |
| | Sulfur metabolism | |
| PF2025 | sulfur-induced protein | 7.1 |
| | Redox balance | |
| **PF1447** | Mbx | 3.1 |
| **PF1451** | Mbx | 3.7 |
| | Transport | |
| PF0360 | ABC transporter | 3.1 |
| | Unknown | |
| **PF1073** | conserved hypothetical protein | 4.2 |
| **PF1074** | conserved hypothetical protein | 4.0 |
| **PF1314** | conserved hypothetical protein | 12.3 |
| **PF1315** | conserved hypothetical protein | 5.1 |
| PF0101 | conserved hypothetical protein | 3.9 |
| **PF1080** | hypothetical protein | 3.3 |
| PF0693 | conserved hypothetical protein | 3.1 |
| | Miscellaneous | |
| PF1518 | thiamin-binding periplasmic protein precursor | 4.7 |
| PF0426 | phosphoribosylaminoimidazole carboxylase | 3.3 |
| PF1967 | spermidine/putrescine binding protein | 3.3 |
| PF1702 | Aspartate aminotransferase | 3.0 |

*Genes of known function*. At 30 min, there is very small number of genes of known function that are up-regulated. The list includes PF0410, which is a methyltransferase involved in recombination, branched chain amino acid synthesis gene PF0936, and sulfur-induced protein PF2025.

*Genes of unknown function*. One of the most striking features of transcriptional response at 30 min of adding CHP is the strong up-regulation of PF0101. It is 41-fold up-regulated as shown by microarray analysis and more than 350-fold up-regulated by q-PCR analysis (Table 3.3). Since this is a conserved hypothetical protein, we do not have enough information about its protection mechanism. However, it is one of the key players in providing protection against CHP-induced oxidative stress.

A comparison of the transcriptional responses at 10 min and 30 min after adding CHP showed that there were 7 ORFs up-regulated at both time points. These are PF0101, PF0642, PF0643, PF1072, PF1080, PF1314 and PF2025. All of these genes except PF1072 are of unknown function and so information about their protection mechanism still needs to be revealed. PF1072 is a proposed oxidative stress regulator in *P. furiosus*. It was found to be regulated by hydrogen peroxide addition as well as in cold shock studies (*15-16*). Q-PCR analysis of PF1072 shows that it is immediately up-regulated to high levels (48.9-fold) within 10 min in response to CHP and then goes back to a very low level within an hour.

Discussion

Table 3.3 represents some of the key genes which are involved in response to CHP. These key genes are either up-regulated more than 3-fold both at 10 or 30 min or they

**Table 3.2.** ORFs whose expression is up-regulated within 30 minutes of adding CHP. Those in bold are potentially part of operons.

| PF # | Gene name | Fold |
|---|---|---|
| | Oxidative stress | |
| **PF1072** | Winged helix repressor DNA binding | 3.6 |
| | CRISPR related | |
| **PF0642** | CRISPR associated protein | 4.0 |
| PF0643 | CRISPR associated protein | 3.1 |
| **PF1126** | CRISPR associated protein | 3.1 |
| **PF1130** | CRISPR associated protein | 3.2 |
| | Sulfur metabolism | |
| PF2025 | sulfur-induced protein | 4.2 |
| | Transport | |
| PF0744 | ABC transporter | 3.9 |
| | Unknown | |
| PF0101 | conserved hypothetical protein | 41.3 |
| **PF1080** | hypothetical protein | 16.4 |
| **PF1314** | conserved hypothetical protein | 6.3 |
| PF0886 | conserved hypothetical | 5.1 |
| PF0479 | hypothetical protein | 4.1 |
| PF1621 | hypothetical protein | 3.9 |
| PF1681 | hypothetical | 3.6 |
| PF0347 | conserved hypothetical protein | 3.6 |
| PF1973 | conserved hypothetical protein | 3.5 |
| PF0926 | hypothetical protein | 3.4 |
| PF1247 | conserved hypothetical protein | 3.2 |
| PF0352 | conserved hypothetical protein | 3.1 |
| PF1463 | hypothetical protein | 3.1 |
| | Miscellaneous | |
| PF0401 | methyltransferase | 9.4 |
| **PF0936** | ketol-acid reductoisomerase | 4.0 |
| PF1713 | carbamoyl-phosphate synthase small subunit | 3.7 |
| PF0456 | cobalt-activated carboxypeptidase | 3.5 |
| PF0925 | heme biosynthesis protein | 3.0 |

**Table 3.3**. Q-PCR analysis of key genes involved in CHP induced oxidative stress.

| PF# | Gene name | Fold | | | |
|---|---|---|---|---|---|
| | | 10 min | 15 min | 30 min | 60 min |
| PF0101 | conserved hypothetical protein | 9 | 153 | 386 | 81 |
| PF1033 | Alkyl hydroperoxide reductase | 108 | 77 | 36 | 21 |
| PF1072 | Winged helix repressor DNA binding | 49 | 24 | 15 | 6 |
| PF1080 | hypothetical protein | 11 | 23 | 73 | 13 |
| PF1193 | Dps-like protein | 18 | 21 | 11 | 2 |
| PF1314 | conserved hypothetical protein | 143 | 130 | 120 | 48 |
| PF2025 | sulfur-induced protein | 29 | 21 | 14 | 5 |

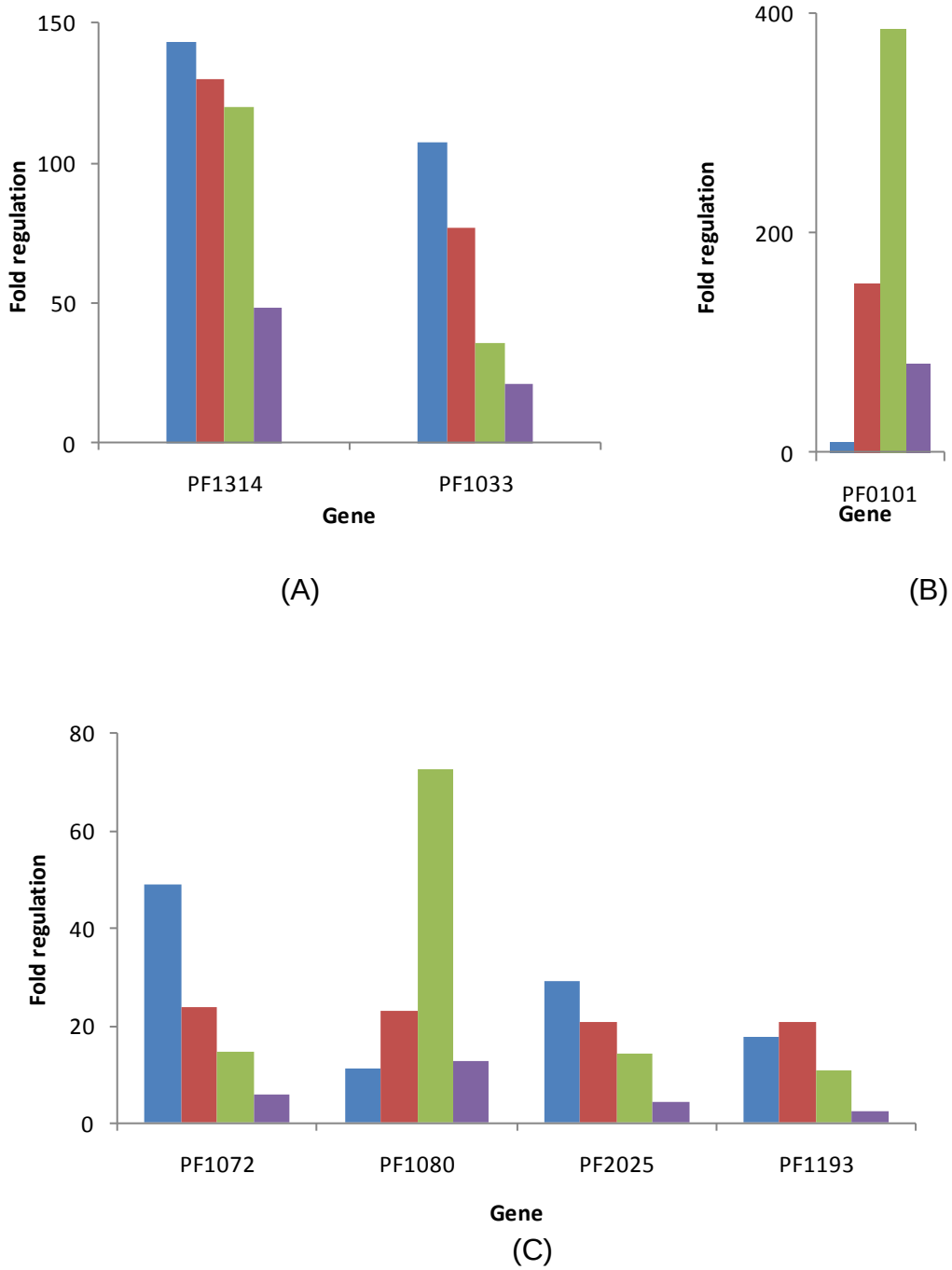



**Fig. 3.3**. Q-PCR analysis of key genes in CHP induced oxidative stress.

Fold regulation at 10min ( ■ ), 15 min ( ■ ), 30 min ( ■ ) and 60 min ( ■ ).

are up-regulated more than 3-fold in response to hydrogen-peroxide induced oxidative stress in *P. furiosus.* One of the most striking conclusions from this study is the significantly high up-regulation of PF0101. This encodes a conserved hypothetical protein and is conserved only among the members of Thermococcales. Homologs are not found in other archaea or in bacteria but are found in *P. horikoshii, P. abyssi and Thermococcus kodakarensis.* Microarray analysis showed that the regulation of PF0101 is time dependent (at 10 min it is 3.9-fold regulated, Table 3.1, whereas after 30 min it is 41.3-fold up-regulated, Table 3.2) with maximum up-regulation at 30 min. This response was confirmed by QPCR analysis, Fig.3.3 (B). PF0101 is also regulated under hydrogen peroxide and cold response in *P. furiosus* (15-16) suggesting that it may be a general stress response gene.

As expected, PF1033, which encodes alkyl hydroperoxide reductase was up-regulated in response to CHP and comprise the inducible response to CHP. This gene was also up-regulated in hydrogen peroxide–induced shock in *P. furiosus*. Homologs of PF1033 are found in a large number of archaea such as *P. horikoshii*, *Thermoplasma volcanium*, *Aeropyrum pernix* and *Sulfolobus solfataricus.* Alkyl hydroperoxide reductase from *P. horikoshii* has also been shown to utilize CHP as a substrate (63). According to Q-PCR analysis, Fig. 3.3 (A), PF1033 is immediately turned “ON” at high levels within 10 minutes of adding CHP and comes to minimal levels after 30 minutes.

PF1072, which encodes a winged helix repressor DNA binding protein, is found to be conserved among members of Thermococcales such as *P. horikoshii*, *P. abyssi* and *Thermococcus kodakarensis*. This gene is also up-regulated more than 3-fold in

hydrogen peroxide-induced stress in *P. furiosus* as well as when *P. furiosus* was exposed to a decrease in temperature (15, 16). The Q-PCR analysis Fig 3.3 (C) indicated that PF1072 is also turned “ON” at high levels within 10 minutes of adding CHP.

PF1080 encodes a conserved hypothetical protein and its homolog is found in *P. abyssi*. This gene is also up-regulated in hydrogen peroxide–induced stress in *P. furiosus*. Q-PCR analysis, Fig 3.3(C), indicated that PF 1080 has maximum up-regulation after 30 minutes of adding CHP.

PF1193 encodes a Dps-like protein. Homologs of this gene are widely spread among bacteria and archaea. This gene is also found to be up-regulated more than 3-fold in hydrogen peroxide-induced stress in *P. furiosus*. Homolog of Dps-like protein in *S. solfataricus* was among the highly expressed genes, when *S. solfataricus* was exposed to 30 µM hydrogen peroxide (27). On comparing the regulation of PF1193 in CHP and hydrogen peroxide–induced stress in *P. furiosus* at 30 min, it was found that PF1193 is not as highly regulated (11-fold) in response to CHP, Fig 3.3 (C) as it is in response to hydrogen peroxide (51.4-fold) (16)

PF1314 encodes a hypothetical protein and is only found in *P. furiosus.* No homologs are found in other *Pyrococcus* species. This gene is also up-regulated in response to hydrogen peroxide-induced stress in *P. furiosus.* Q-PCR analysis, Fig 3.3 (A), indicated that PF1314 is also immediately turned “ON” at high levels (144-fold) within 10 minutes of adding CHP, and remains significantly up-regulated (120-fold) until 30 minutes after adding CHP.

PF2025 encodes the sulfur induced protein, SipA. It is highly up-regulated when *P. furiosus* is grown in presence of sulfur. In addition, it is also highly up-regulated (18.8-fold as indicated by microarray analysis) by hydrogen peroxide induced stress in *P. furiosus.* Close homologs are found in *P. abyssi*, *P. horikoshii* and *Thermococcus kodakarensis.* Q-PCR analysis, Fig 3.3 (C), indicated that PF2025 has maximum up-regulation at 10 minutes after adding CHP.

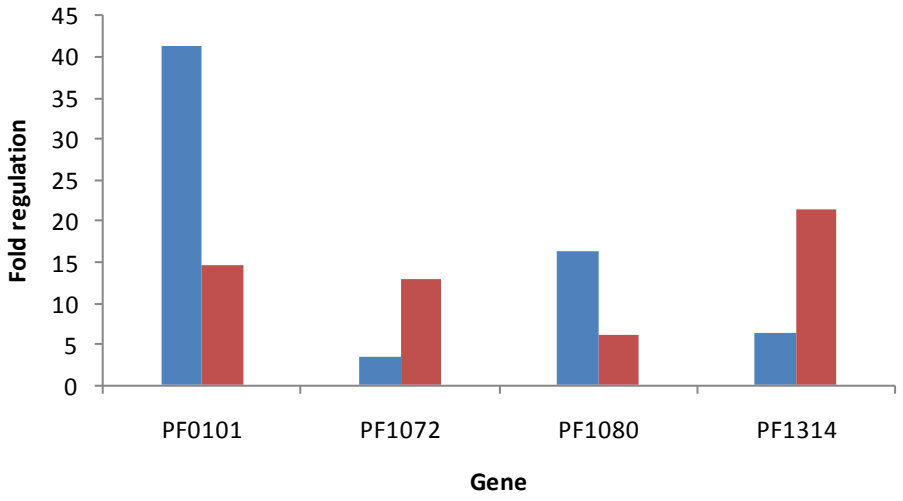
Genes involved in the SOR-reaction pathway were not regulated. These include superoxide reductase (PF1281), rubredoxin (PF1282), rubrerythrin (PF1283) and NADPH rubredoxin oxidoreductase (PF1197). None of these genes were significantly regulated in response to CHP shock. Microarray analysis has revealed that when *P. furiosus* is grown under non-oxidative stress conditions, these genes are amongst the most highly expressed genes (14). None of these genes were regulated in hydrogen peroxide induced oxidative stress. This suggests that these genes are constitutively expressed at high levels and protects the organism against oxidative stress.

On comparing transcriptional response of *P. furiosus* to CHP and hydrogen peroxide, 30 min after adding the oxidant, 10 genes were found to be up-regulated in both cases (Table 3.4). The list mainly includes genes of unknown function which impedes us in understanding the protection mechanism. One of the differences in transcriptional responses to CHP and hydrogen peroxide is the significantly high up-regulation of PF1193 encoding Dps-proteins at 30 min of adding hydrogen peroxide, Fig 3.4(B). PF1193 was found to be 12.9-fold regulated in hydrogen peroxide shock but was less than 3-fold regulated in CHP shock as indicated by microarray analysis.

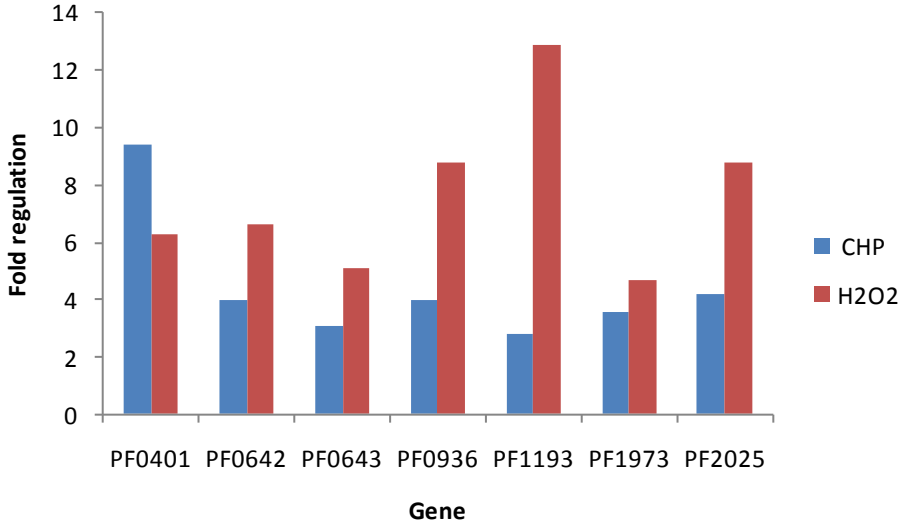
In summary, the overall response to CHP can be divided into the constitutive and the inducible response. The constitutive response includes genes from the SOR pathway while the inducible response includes PF1033 encoding alkyl hydroperoxide reductase. Characterization of genes of unknown function like PF0101, PF1072, PF1080, PF1314. and PF1973 will give us a better understanding of the possible roles of these proteins in oxidative stress defense in *P. furiosus*. Fig. 3.5 summarizes the results obtained from this study.

**Table 3.4.** Comparison of transcriptional responses to 20 µM CHP and 500 µM $H_2O_2$ in *P. furiosus.* Those in bold are potentially part of operons.

| PF # | Gene name | Fold CHP | Fold $H_2O_2$ |
|---|---|---|---|
| | oxidative stress | | |
| **PF1072** | Winged helix repressor DNA binding | 3.6 | 13.0 |
| | CRISPR associated | | |
| **PF0642** | CRISPR associated protein | 4.0 | 6.6 |
| PF0643 | CRISPR associated protein | 3.1 | 5.1 |
| | sulfur metabolism | | |
| PF2025 | sulfur-induced protein | 4.2 | 8.8 |
| | unknown | | |
| PF0101 | conserved hypothetical protein | 41.3 | 14.6 |
| **PF1080** | hypothetical protein | 16.4 | 6.1 |
| **PF1314** | conserved hypothetical protein | 6.3 | 21.5 |
| PF1973 | conserved hypothetical protein | 3.5 | 4.7 |
| | miscellaneous | | |
| PF0401 | methyltransferase | 9.4 | 6.3 |
| PF0936 | ketol-acid reductoisomerase | 4.0 | 8.8 |




(A)




(B)

**Fig. 3.4.** Comparison of transcriptional responses to 20 µM CHP and 500 µM $H_2O_2$ in *P. furiosus.* Genes regulated by CHP (■) and hydrogen peroxide (■).

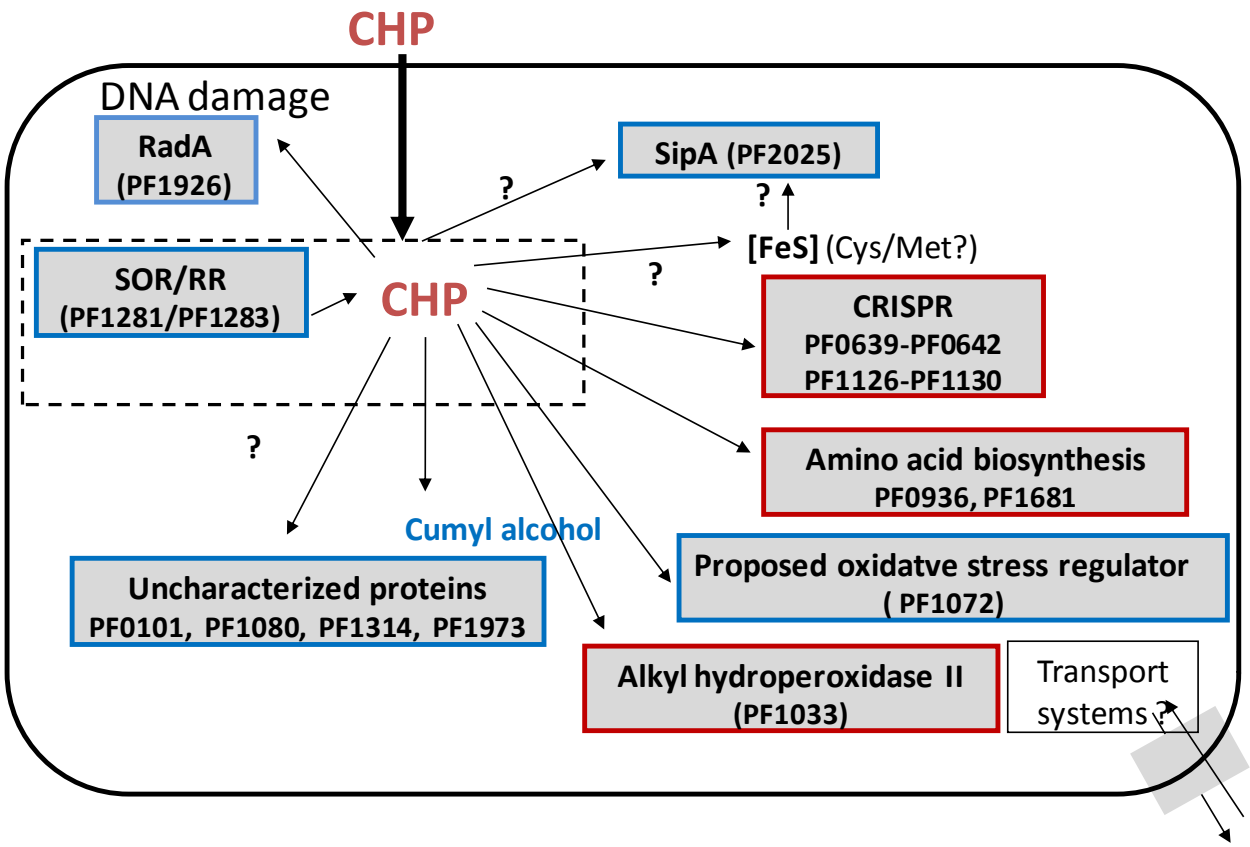



**Fig. 3.5.** Proposed model for oxidative stress response of CHP in *P. furiosus,* adapted from (16). The constitutive defense is boxed (dotted line) and the rest of the proteins are up-regulated in response to CHP. Genes induced by CHP only are in red boxes .Genes common in CHP and $H_2O_2$ shock are in blue box.

CHAPTER 4

## SUMMARY

*Pyrococcus furiosus* is an obligate anaerobe that inhabits shallow marine hydrothermal vents. In its natural environment, when these hot vent fluids get mixed with cold and oxygenated sea water, *P. furiosus* is exposed to oxidative stress. A SOR pathway has been proposed to be involved in protecting *P. furiosus* against oxidative stress (53, 56). In this pathway, SOR reduces superoxide to hydrogen peroxide. It has been proposed that a redox protein rubredoxin (Rd) provides electrons to SOR, which in turn is reduced by the enzyme NADPH:rubredoxin oxidoreductase (NROR). NROR utilizes NADPH as an electron donor. Hydrogen peroxide generated by SOR is reduced to water by a non heme iron protein rubrerythrin (Rr) (21, 73). The proteins involved in SOR pathway have been isolated and purified individually by multiple chromatographic columns (21, 51, 53, 56, 59). In this study it was investigated if any of the proteins involved in SOR pathway SOR, Rd, NROR and Rr directly interact with each other. Polyclonal antibodies were raised in rabbits against recombinant forms of SOR, Rd, NROR and Rr. The antisera obtained were used for immunoprecipitation analysis. Soluble fraction of *P. furiosus* cell extract (S100) was analyzed by these antisera. Antisera obtained from recombinant SOR and recombinant Rr were found to be specific as indicated by a single band at the expected molecular weight of each protein. Immunoprecipitation analysis as reported in Chapter 2, suggested that SOR and Rr interact with each other. In order to determine

the size of the complex formed by SOR and Rr, size exclusion chromatography using Superdex 200 was performed. S100 from *P. furiosus* was analyzed by size exclusion chromatography. The proteins eluted from the column were analyzed by immunoblotting using SOR antiserum and Rr antiserum. Immunoblotting results suggested that SOR and Rr form 1:1 complex of 96 kDa and may also form a 2:1 complex of 192 kDa. A similar size exclusion chromatographic experiment was performed with recombinant SOR, recombinant Rr individually and mixed in 2:1 ratio. Immunoblot analysis of the SOR and Rr mixture suggested that pure SOR and pure Rr do not interact with each other. However, only a small part of total SOR and Rr content in the cell is involved in forming the complex and most of it remains in its holoenzyme form. Homologs of SOR and Rr have been reported in a large number of anaerobic archaea and bacteria examined (57, 60-62, 74). It has not been reported to date if SOR and Rr interact in any other organism. This is the first report where an interaction between SOR and Rr has been reported.

Oxidative stress has been reported in various aerobic and anaerobic archaea (16, 27, 65-67). There have been no reports so far to study oxidative stress on a genome wide basis using organic peroxide in any archaeal species. One of the other goals of this study was to study the transcriptional response to cumene hydroperoxide (CHP) on a genome wide basis in the hyperthermophilic archaeon *P. furiosus.* From the work reported in chapter 3, a 20 liter culture of *P. furiosus* was shocked with 20 µM CHP when the cells were in mid log phase. Microarray analysis were done using RNA prepared from *P. furiosus* cells removed at 10 and 30 min after adding CHP and

compared with RNA prepared from *P. furiosus* cells removed just before adding CHP. The microarray analysis suggested that there were 50 genes whose expression was up-regulated more than 3-fold in response to CHP. Out of those 50 genes that were up-regulated, there were 29 genes of unknown function. This suggests that a large number of genes of unknown functions were regulated in response to CHP. One of the most striking conclusions from the response to CHP is the strong up-regulation of a conserved hypothetical gene PF0101. This gene was up-regulated more than 40-fold within 30 minutes of adding CHP as indicated by microarray analysis. However, the same gene was found to be 386-fold regulated by q-PCR analysis. Overall, the response to CHP can be divided into constitutive and the inducible response. The constitutive response includes the genes involved in SOR pathway. The inducible response includes genes encoding alkyl hydroperoxide reductase PF1033, the gene encoding SipA PF2025. A large number of conserved hypothetical proteins such as PF0101, PF1072, PF1080, PF1314 and PF1973 also comprise the inducible response. As suggested earlier, most of the genes regulated in response to CHP are of unknown function. These proteins will need to be characterized in order to obtain an insight as how these proteins are involved in protecting *P. furiosus* against CHP induced oxidative stress. This is also the first report where the effect of CHP has been studied on a genome-wide basis.

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